Plant Physiol. (1994) 106: 109-118

The Cloning of Two Tomato Lipoxygenase Genes and Their Differential Expression during Fruit Ripening[1]

Bonita J. Ferrie, Nathalie Beaudoin, William Burkhart, Caroline G. Bowsher[2], and Steven J. Rothstein*

Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (B.J.F., N.B., C.G.B., S.J.R.); and Glaxo Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709 (W.B.)

A membrane-associated lipoxygenase from breaker-stage fruit of tomato (*Lycopersicon esculentum* Mill.) was purified and partially sequenced. Using degenerate oligonucleotides corresponding to portions of this sequence, a cDNA was amplified by PCR and used to screen a breaker fruit cDNA library. Two clones, *tomloxA* and *tomloxB*, were isolated and one of these (*tomloxA*) corresponded to the isolated protein. Genomic clones were isolated and sequence data from these were used to obtain the 5′ ends of the cDNAs. The 2.8-kb cDNAs encode proteins that are similar in size and sequence to each other and to other plant lipoxygenases. DNA blot analysis indicated that tomato contains three or more genes that encode lipoxygenase. RNA blot analysis showed that *tomloxA* is expressed in germinating seeds as well as in ripening fruit, where it reached its peak during breaker stage. *tomloxB* appears to be fruit specific and is at its highest level in ripe fruit.

Lipoxygenases (EC 1.13.11.12) comprise a class of iron-containing enzymes that use molecular oxygen in the dioxygenation of fatty acids containing a 1,4-pentadiene structure such as linoleic and linolenic acid in plants and arachidonic acid in mammalian cells. The resulting lipid hydroperoxides are further metabolized into physiologically active compounds. In mammals, some of these bioregulatory molecules produced are leukotrienes, prostaglandins, lipoxins, and thromboxanes (Yamamoto, 1992). The lipoxygenase products in higher plants are metabolized by one of two major pathways leading to the formation of jasmonic acid or traumatin (wound hormone), which are known to have regulatory functions (Hildebrand, 1989; Siedow, 1991).

There is growing evidence that plant lipoxygenases are involved in several physiological functions including growth and development (Kato et al., 1992; Matsui et al., 1992; Ohta et al., 1992), defense against wounding and pathogens (Bell and Mullet, 1991; Croft et al., 1993; Fournier et al., 1993; Melan et al., 1993), and senescence (reviewed by Paliyath and Droillard, 1992). Senescence and fruit ripening are characterized by an early deterioration of cellular membranes. It is not known whether plant lipoxygenases can directly attack membrane fatty acids (Hildebrand, 1989), but most plant lipoxygenases, including a membrane-associated tomato (*Lycopersicon esculentum* Mill.) fruit lipoxygenase (Todd et al., 1990), prefer free fatty acid substrate. It is proposed that after the release of free fatty acids from the membrane by lipases, lipoxygenases catalyze lipid peroxidation and loss of membrane phospholipid and fatty acid (Thompson, 1988). During the lipoxygenase reaction, free radicals are formed (Lynch and Thompson, 1984; Paliyath and Droillard, 1992). These derivatives and the lipid hydroperoxides can exert deleterious effects on membranes and proteins and may contribute to the loss of membrane function accompanying fruit ripening and senescence (Todd et al., 1990).

To determine the physiological functions of lipoxygenases, more must be learned about their cellular and subcellular localization. Although most subcellular compartments of higher plant cells have been identified at one time or another as lipoxygenase sites, the results have been inconclusive and often contradictory (Mack et al., 1987). Most lipoxygenases that have been well characterized are soluble enzymes found in the cytoplasm (Siedow, 1991). Immunochemical labeling and fractionation studies have revealed membrane association of lipoxygenase in plant and animal cells (Vick and Zimmerman, 1987; Yamamoto, 1992). In mammalian cells, one type of lipoxygenase is known to oxygenate esterified polyenoic fatty acids that include those that are part of complex substrates such as biomembranes and lipoproteins (Schewe and Kuhn, 1991). Another form of mammalian lipoxygenase is found in the cytosol but is rapidly translocated to a membrane compartment when activated. The lipoxygenase associates with an integral membrane protein, FLAP, which has recently been shown to bind arachidonic acid (Mancini et al., 1993). It is thought that after release from membrane phospholipids by phospholipases, this substrate is transferred by FLAP to the enzyme.

In plant cells, membrane-associated lipoxygenases have recently been reported for senescing carnation petals (Rouet-Mayer et al., 1992), tomato fruit (Todd et al., 1990; Bowsher et al., 1992), and cucumber and soybean cotyledons (Feußner and Kindl, 1992). The cucumber and soybean proteins were found to be localized in the lipid body membranes, where previously soybean lipoxygenase had not been found. It is

[1] This work was supported by a grant from the Natural Sciences and Engineering Research Council to S.J.R.

[2] Present address: Plant Metabolism Research Unit, Department of Cell and Structural Biology, University of Manchester, Williamson Building, Oxford Road Manchester, M13 9PL, United Kingdom.

* Corresponding author; fax 1-519-837-2075.

Abbreviations: FLAP, 5-lipoxygenase-activating protein; RACE, rapid amplification of cDNA ends.

interesting that this lipoxygenase is found in lipid bodies that contain fatty acid in the form of triglycerides and are known to lack lipase activity (Feußner and Kindl, 1992).

To investigate the role of lipoxygenase in ripening, senescence, and membrane turnover, we have purified a membrane-associated lipoxygenase from tomato fruit (Bowsher et al., 1992). In this paper we report the partial amino acid sequencing of this protein and the isolation of the corresponding gene. We also discuss another isolated tomato fruit lipoxygenase gene and the characterization and expression of both.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill.) plants were grown under standard greenhouse conditions. Pericarp tissue from breaker-stage fruit (cv Caruso) was used for protein purification and isolation of RNA for construction of the cDNA library and PCR amplification. RNA for blot analysis of four stages of fruit ripening was isolated from the pericarp of cv Rutgers. Root, leaf, and seed RNA was isolated from cv Jumbo. Leaf DNA for genomic blot analysis and library construction was isolated from cvs Ailsa Craig and Caruso, respectively. For RNA extraction, seeds were surface sterilized and allowed to imbibe in sterile water for 24 h in the dark at room temperature. Germinated seeds were left another 6 d (2 d postgermination). All tissue was either used immediately after harvest or frozen in liquid nitrogen and stored at −80°C.

Amino Acid Sequencing

Membrane-associated lipoxygenase was purified as described by Bowsher et al. (1992). Protein digestion with endoproteinase Lys-C (Wako Chemicals, Dallas, TX) was performed in 0.1 M Tris-HCl, pH 8.5, containing 2 M guanidine-HCl for 18 h at room temperature with an enzyme:substrate ratio of 1:50. The protein was incubated at 50°C for 30 min in 6 M guanidine-HCl before the addition of protease. The Lys-C peptides were isolated on an Aquapore OD300 (1 × 250 mm; Brownlee) column employing a linear gradient of 0 to 48% acetonitrile in 0.1% TFA over 90 min with a flow rate of 100 μL/min. Automated Edman sequencing was performed using the ABI 477A liquid-pulse sequencer, and phenylthiohydantoin amino acids were identified on an ABI 120A (Applied Biosystems, Inc., Santa Clara, CA) phenylthiohydantoin analyzer. The amino acid sequences were compared to known plant lipoxygenase sequences (GenBank) and this information was used to construct two degenerate oligonucleotides.

PCR Amplification of cDNA Probe

Following the mixed oligonucleotide primed amplification of cDNA procedure of Lee and Caskey (1990), total RNA (20 μg) from breaker fruit and the antisense oligonucleotide primer were used to synthesize first-strand cDNA. This was then used in a PCR with both degenerate primers following the recommended conditions except for an annealing temperature of 37°C. The PCR product was cloned into pBluescript (Stratagene, La Jolla, CA).

Construction and Screening of cDNA Library

Poly(A)$^+$ RNA was purified from breaker-stage fruit using an oligo(dT) column (Sambrook et al., 1989). Five micrograms was used to construct a cDNA library in λgt11 using the λ Librarian System (Invitrogen, San Diego, CA) and Gigapack (Stratagene). Approximately 56,000 phage were screened using the plaque lift methods of Sambrook et al. (1989) and the 830-bp lipoxygenase PCR fragment as a probe, which was gel purified and labeled by random priming (Feinberg and Vogelstein, 1983). Filters were washed with increasing stringency to 0.2× SSC, 0.1% SDS at 65°C. DNA was isolated from three positive clones using either Lambdasorb (Promega, Madison, WI) or PCR amplification with primers specific to the λ vector. The inserts were released by digestion with *Not*I and cloned into pBluescript.

Construction and Screening of Genomic Library

Genomic DNA was extracted from tomato leaves as described by Goring et al. (1992) and used to make a genomic library in λ FixII using the Stratagene λ FixII and Gigapack II Gold kits. More than 300,000 phage were transferred to nylon membranes according to Sambrook et al. (1989). Membranes were prewashed in 5× SSC, 0.5% SDS, and 1 mM EDTA (pH 8.0) for 30 min at 42°C and screened with random-primed labeled probes consisting of either a 1.4-kb fragment from the 3′ end of *tomloxA* cDNA or a 0.8-kb fragment from *tomloxB* cDNA. Prehybridization of the membranes was carried out at 42°C in 5× SSPE, 10× Denhardt's solution, and 0.5% SDS for 3 h. Hybridization was overnight at 42°C in 50% formamide, 5× SSPE, and 0.5% SDS. Filters were washed twice for 15 min in 2× SSC, 0.1% SDS at room temperature and twice for 30 min in 0.1× SSC, 0.1% SDS at 60°C before autoradiography. Positive plaques were picked and purified and DNA was extracted using the plate lysate method of Sambrook et al. (1989). Clones were analyzed by restriction mapping and several restriction fragments or whole inserts were subcloned into pBluescript and sequenced.

PCR Amplification of 5′ cDNAs

The 5′ end of *tomloxA* was isolated using the RACE procedure (Froham, 1990) with modifications and adapter primers as outlined by Goring et al. (1992). First-strand cDNA was synthesized and polyadenylated according to Harvey and Darlison (1991) using 20 μg of total RNA from breaker-stage fruit. Of the 100 μL of tailed cDNA, 1 μL was amplified in a 100-μL reaction using 50 nM each of the dT_{17} adapter primer and a gene-specific primer for 4 cycles of 94°C for 1 min, 34°C for 30 s, and 72°C for 30 s. After 4 cycles the adapter primer and a gene-specific primer 5′ to the first one were added to 250 nM at 72°C. The amplification was continued for 45 cycles with the annealing temperature increased to 62°C. One-half of the product was electrophoresed on a 1.7% low-melting-point agarose gel and five plugs were removed from the resulting smeary band with Pasteur pipettes (Zintz and Beebe, 1991). The plugs were heated to 72°C for 10 min and subjected to 40 cycles of PCR using 300 nM each of the adapter primer and the second specific primer and an annealing temperature of 62°C. The resulting single band was purified and cloned into pBluescript. The 5′ end of *tomloxB* was amplified using an antisense primer specific to the partial cDNA clone (and downstream of several introns) and a 5′ sense primer deduced from the corresponding genomic clone. The cDNA (1 μL) prepared for 5′ RACE was used in a 100-μL reaction containing 200 nM of each primer, 200 μM each dNTP, and 2.5 units of *Taq* polymerase. The PCR conditions were 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min for a total of 30 cycles. The product was cloned into pBluescript.

DNA Extraction and Blot Analysis

Genomic DNA was extracted from tomato leaves as for the library. Approximately 10 μg of DNA was cleaved with *Eco*RI and *Hin*dIII (BRL). After fractionation on a 0.7% agarose gel, DNA was transferred to Zetabind membrane (Cuno Inc., Meriden, CT) in 20× SSC. The dry membrane was prewashed in 0.1× SSC, 0.5% SDS at 60°C for 30 min. Prehybridization of the membrane was as for genomic plaque lifts. Hybridization was overnight at 42°C in 50% formamide, 5× SSPE, 10% dextran sulfate, 0.5% SDS, and 50 μg μL^{-1} salmon sperm DNA. The first probe used was a 1.5-kb gel-purified fragment from the 3′ end of *tomloxB* cDNA. After hybridization, the membrane was washed twice for 30 min in 2× SSC, 0.1% SDS at room temperature and twice for 30 min in 2× SSC, 0.1% SDS at 60°C before autoradiography. Higher-stringency washes were also performed for 30 min in 0.1× SSC, 0.1% SDS at 60°C and at 65°C, with each wash being followed by autoradiography of the membrane. The membrane was stripped of probe according to the Zetabind manufacturer and prehybridized and hybridized as above using as probe a 1.4-kb fragment from the 3′ end of *tomloxA* cDNA. Washes were performed as described.

RNA Extraction and Blot Analysis

RNA for blot analysis of fruit stages was extracted from pericarp tissue as outlined by Rastogi et al. (1993). The method of Chang et al. (1993) was used to extract RNA from other tissues. RNA (20 μg) was fractionated through a 1.2% formaldehyde gel (Sambrook et al., 1989) and transferred to Zetabind membrane. The membranes were baked at 80°C for 1 to 2 h and prewashed in 0.1× SSC, 0.5% SDS at 60°C for 1 h. Prehybridization was in 5× SSPE, 10× Denhardt's solution, 0.5% SDS at 42°C for 3 h, and hybridization was in 5× SSPE, 0.5% SDS, 50% formamide, 10% dextran sulfate, and 50 μg μL^{-1} salmon sperm DNA at 42°C overnight. Membranes were probed with full-length cDNAs of *tomloxA* and *tomloxB* and washed under high-stringency conditions of 0.1× SSC, 0.1% SDS at 65°C. Equal loading of RNA samples was determined by probing with p318, which contains rRNA genes from wheat (S. Rothstein, unpublished data), and washing in 0.2× SSC, 0.1% SDS at 55°C. The blots were stripped of probe between hybridizations.

DNA Sequencing and Analysis

Nucleotide sequences were determined by dideoxy sequencing using the Sequenase enzyme (United States Biochemical, Cleveland, OH). To avoid errors that may have occurred during PCR amplification of the 5′ cDNA clones, either three clones from separate PCR reactions were sequenced or the sequence was compared to the genomic DNA, where it was available. The cDNA clones were sequenced using restriction fragments and specific primers. The genomic clones were deleted using exonuclease III and mung bean nuclease using the procedure recommended in the Stratagene kit. The softwares DNASIS and PROSIS (Hitachi Software Engineering America Ltd., Brisbane, CA) were used for nucleic acid and protein sequence analysis, respectively.

RESULTS

Protein Sequencing and Isolation of cDNA Probe

A membrane-associated lipoxygenase was isolated from breaker-stage tomato fruit. This enzyme was kinetically distinguishable from the soluble form and Triton X-100 (2%, v/v) was required to solubilize its activity after sedimentation with the microsomal membranes (Bowsher et al., 1992). This enzyme was subsequently digested with endoproteinase and the resulting fragments were purified and sequenced. Amino acid sequences of the four resulting peptides (underlined in Fig. 1a) were compared to the plant lipoxygenase sequences (pea and soybean) available from GenBank and to a partial tobacco sequence (Bookjans et al., 1988). Three of the peptides showed a high level of similarity to the legume (68–89% identity) and tobacco (78–100% identity) sequences. The fourth peptide (amino acids 245–260, Fig. 1a) showed 94% identity with the tobacco sequence but no similarity to pea or soybean lipoxygenases. N-terminal sequencing of the tomato lipoxygenase was not possible due to blockage of the N-terminal amino acid.

Two regions of low degeneracy were chosen to design two 17-bp degenerate oligonucleotides (shaded in Fig. 1a). These were used as primers to amplify cDNA synthesized from RNA of breaker tomato fruit. A single band of around 830 bp was produced and, based on the known plant sequences, a band of approximately 800 bp was expected. This product was cloned and partially sequenced. The DNA sequence obtained showed 57 to 63% identity with the legume lipoxygenase sequences and the translation of the ends of the clone predicted amino acid sequences with 100% identity to the isolated peptides. This result indicated that it was a partial cDNA for tomato lipoxygenase.

Isolation of Lipoxygenase cDNAs

A breaker-stage tomato pericarp cDNA library was constructed in λgt11 and screened with the 830-bp PCR product. Of the three clones isolated, two were found to be identical after partial sequencing. The larger of the two (1.8 kb) and the third clone (2 kb) were fully sequenced. Both of these clones were found to be similar to other plant lipoxygenase genes, with overall nucleic acid sequence identity values ranging from approximately 60 to 70%. The 1.8-kb clone was found to code for amino acids that corresponded exactly with sequence from the purified membrane-associated protein. This clone was designated *tomloxA* and the second clone was named *tomloxB*.

a

```
G ACA ACA AAA ACT CAT CAT ATC TTT TTA TAC TTT TTA ATA TTT CTC TTA TTC ATC ATC ATG TTA GGT CAA CTT GTG GGT GGA    82
                                                                              M   L   G   Q   L   V   G   G      8

TTA ATT GGT GGC CAT CAT GAC TCA AAG AAA GTT AAA GGA ACT GTG GTG ATG ATG AAG AAA AAT GCT CTA GAT TTT ACT GAT CTT   166
 L   I   G   G   H   H   D   S   K   K   V   K   G   T   V   V   M   M   K   K   N   A   L   D   F   T   D   L    36

GCT GGC TCT TTG ACT GAT AAA ATC TTT GAA GCC CTT GGC CAA AAG GTT TCT TTT CAA TTA ATT AGT TCT GTT CAA AGT GAT CCT   250
 A   G   S   L   T   D   K   I   F   E   A   L   G   Q   K   V   S   F   Q   L   I   S   S   V   Q   S   D   P    64

GCA AAT GGC TTA CAA GGG AAA CAT AGT AAT CCA GCC TAT TTG GAG AAC TTT CTC CTT ACT CTA ACA CCA TTA GCA GCT GGT GAA   334
 A   N   G   L   Q   G   K   H   S   N   P   A   Y   L   E   N   F   L   L   T   L   T   P   L   A   A   G   E    92

ACA GCC TTT GGT GTC ACA TTT GAT TGG AAT GAG GAG TTT GGA GTT CCA GGT GCA TTT GTC ATA AAA AAT ATG CAT ATC AAT GAG   418
 T   A   F   G   V   T   F   D   W   N   E   E   F   G   V   P   G   A   F   V   I   K   N   M   H   I   N   E   120

TTC TTT CTC AAG TCA CTC ACA CTT GAA GAT GTG CCT AAT CAT GGC AAG GTC CAT TTT GTT TGC AAT TCT TGG GTT TAT CCT TCT   502
 F   F   L   K   S   L   T   L   E   D   V   P   N   H   G   K   V   H   F   V   C   N   S   W   V   Y   P   S   148

TTT AGA TAC AAA TCA GAT AGA ATT TTC TTT GCA AAT CAG CCA TAT CTC CCA AGT GAA ACA CCA GAG CTT TTG CGA AAA TAC AGA   586
 F   R   Y   K   S   D   R   I   F   F   A   N   Q   P   Y   L   P   S   E   T   P   E   L   L   R   K   Y   R   176

GAA AAT GAA TTG GTA ACA TTA AGA GGA GAT GGA ACT GGA AAG CGC GAG GCG TGG GAT AGG ATT TAT GAC TAT GAT GTC TAC AAT   670
 E   N   E   L   V   T   L   R   G   D   G   T   G   K   R   E   A   W   D   R   I   Y   D   Y   D   V   Y   N   204

GAC TTA GGT AAT CCT GAT CAA GGT AAA GAA AAT GTT AGA ACT ACC TTA GGA GGT TCT GCT GAC TAC CCG TAT CCT CGG AGA GGA   754
 D   L   G   N   P   D   Q   G   K   E   N   V   R   T   T   L   G   G   S   A   D   Y   P   Y   P   R   R   G   232

AGA ACT GGT AGA CCA CCA ACA CGA ACA GAT CCT AAA AGT GAA AGC AGG ATT CCA CTT ATT CTG AGC TTA GAC ATC TAT GTA CCG   838
 R   T   G   R   P   P   T   R   T   D   P   K   S   E   S   R   I   P   L   I   L   S   L   D   I   Y   V   P   260

AGA GAC GAG CGT TTT GGT CAC TTG AAG ATG TCA GAC TTC CTA ACA TAT GCA TTG AAA TCC ATT GTT CAA TTC ATC CTC CCT GAA   922
 R   D   E   R   F   G   H   L   K   M   S   D   F   L   T   Y   A   L   K   S   I   V   Q   F   I   L   P   E   288

TTA CAT GCC TTG TTT GAT GGT ACC CCT AAC GAG TTC GAT AGT TTT GAG GAT GTA CTT AGA CTA TAT GAA GGA GGG ATC AAA CTT  1006
 L   H   A   L   F   D   G   T   P   N   E   F   D   S   F   E   D   V   L   R   L   Y   E   G   G   I   K   L   316

CCT CAA GGA CCT TTA TTT AAA GCT CTT ACT GAT GCT ATT CCT CTA GAG ATG ATA AGA GAA CTC CTT CGA ACA GAC GGT GAA GGA  1090
 P   Q   G   P   L   F   K   A   L   T   D   A   I   P   L   E   M   I   R   E   L   L   R   T   D   G   E   G   344

ATA TTG AGA TTT CCA ACT CCT CTA GTG ATA AAA GAT AGT AAA ACC GCG TGG AGG ACT GAT GAA GAA TTT GCA AGA GAA ATG CTA  1174
 I   L   R   F   P   T   P   L   V   I   K   D   S   K   T   A   W   R   T   D   E   E   F   A   R   E   M   L   372

GCT GGA GTT AAT CCT GTT ATA ATT AGT AGA CTT GAA GAA TTT CCT CCA AAA AGC AAG CTA GAT CCT GAA CTA TAT GGA AAT CAA  1258
 A   G   V   N   P   V   I   I   S   R   L   E   E   F   P   P   K   S   K   L   D   P   E   L   Y   G   N   Q   400

AAC AGT ACA ATT ACT GCA GAA CAC ATA GAG GGT AAG CTG GAT GGA CTA ACG ATT GAT GAG GCG ATC AAC AGT AAT AAA CTT TTC  1342
 N   S   T   I   T   A   E   H   I   E   G   K   L   D   G   L   T   I   D   E   A   I   N   S   N   K   L   F   428

ATA TTG AAC CAT CAT GAT GTT CTT ATA CCA TAT TTG AGG AGG ATA AAC ACT ACA ACA ACG AAA ACA TAT GCC TCG AGA ACT TTG  1426
 I   L   N   H   H   D   V   L   I   P   Y   L   R   R   I   N   T   T   T   T   K   T   Y   A   S   R   T   L   456

CTA TTC TTG CAA GAT AAT GGA TCT TTG AAG CCA CTA GCA ATT GAA TTG AGT TTG CCA CAT CCA GAT GGA GAT CAA TTT GGT GTT  1510
 L   F   L   Q   D   N   G   S   L   K   P   L   A   I   E   L   S   L   P   H   P   D   G   D   Q   F   G   V   484

ACT AGT AAA GTG TAT ACT CCA AGT GAT CAA GGT GTT GAG GGC TCT ATC TGG CAA TTG GCT AAA GCT TAT GTT GCG GTG AAT GAC  1594
 T   S   K   V   Y   T   P   S   D   Q   G   V   E   G   S   I   W   Q   L   A   K   A   Y   V   A   V   N   D   512

TCT GGT GTT CAT CAA CTG ATT AGT CAT TGG TTG AAT ACA CAT GCT GTG ATC GAG CCA TTT GTG ATT GCA ACA AAC AGG CAA CTA  1678
 S   G   V   H   Q   L   I   S   H   W   L   N   T   H   A   V   I   E   P   F   V   I   A   T   N   R   Q   L   540
                                 *               *

AGT GTG CTT CAC CCT ATT CAT AAG CTT CTA TAT CCT CAT TTC CGG GAC ACA ATG AAT ATT AAT GCT TTG GCA AGA CAG ATC CTA  1762
 S   V   L   H   P   I   H   K   L   L   Y   P   H   F   R   D   T   M   N   I   N   A   L   A   R   Q   I   L   568

ATC AAT GCT GGT GGA GTT CTT GAG AGT ACA GTT TTC CCT TCC AAA TTT GCC ATG GAA ATG TCA GCT GTC GTT TAC AAA GAC TGG  1846
 I   N   A   G   G   V   L   E   S   T   V   F   P   S   K   F   A   M   E   M   S   A   V   V   Y   K   D   W   596

GTC TTC CCT GAT CAA GCC CTT CCA GCT GAT CTT GTT AAG AGG GGA GTA GCA GTT GAG GAC TCG AGT TCT CCT CAT GGT GTT CGT  1930
 V   F   P   D   Q   A   L   P   A   D   L   V   K   R   G   V   A   V   E   D   S   S   S   P   H   G   V   R   624

TTA CTG ATA GAT GAC TAT CCA TAC GCT GTT GAT GGC TTA GAA ATC TGG TCT GCA ATC AAA AGT TGG GTG ACA GAT TAC TGC AGT  2014
 L   L   I   D   D   Y   P   Y   A   V   D   G   L   E   I   W   S   A   I   K   S   W   V   T   D   Y   C   S   652

TTC TAC TAC GGA TCG AAT GAA GAG ATT TTG AAA GAC AAT GAA CTA CAA GCG TGG TGG AAG GAA GTC CGA GAA GTG GGA CAT GGT  2098
 F   Y   Y   G   S   N   E   E   I   L   K   D   N   E   L   Q   A   W   W   K   E   V   R   E   V   G   H   G   680

GAC AAG AAA AAT GAA CCA TGG TGG GCT GAA ATG GAA ACA CCA CAA GAG CTA ATC GAT TCG TGT ACA ACC ATC ATC TGG ATA GCT  2182
 D   K   K   N   E   P   W   W   A   E   M   E   T   P   Q   E   L   I   D   S   C   T   T   I   I   W   I   A   708

TCT GCA CTT CAT GCA GCA GTC AAT TTC GGG CAG TAT CCT TAT GCA GGT TAC CTC CCA AAT CGT CCC ACA GTA AGT CGA AAA TTC  2266
 S   A   L   H   A   A   V   N   F   G   Q   Y   P   Y   A   G   Y   L   P   N   R   P   T   V   S   R   K   F   736
             *

ATG CCT GAA CCA GGT ACT CCT GAA TAC GAA GAG CTA AAG AAA AAC CCC GAT AAG GCA TTC TTG AAA ACC ATC ACA GCG CAG TTA  2350
 M   P   E   P   G   T   P   E   Y   E   E   L   K   K   N   P   D   K   A   F   L   K   T   I   T   A   Q   L   764

CAA ACA TTG CTT GGT GTT TCC CTC ATA GAG ATA TTG TCA AGG CAT ACC ACA GAT GAG ATA TAC CTC GGA CAA CGA GAG TCT CCT  2434
 Q   T   L   L   G   V   S   L   I   E   I   L   S   R   H   T   T   D   E   I   Y   L   G   Q   R   E   S   P   792

GAA TGG ACA AAG GAC AAA GAA CCA CTC GCT GCT TTC GAA AGA TTT GGA AAT AAG TTA ACA GAC ATT GAA AAA CAG ATT ATG CAG  2518
 E   W   T   K   D   K   E   P   L   A   A   F   E   R   F   G   N   K   L   T   D   I   E   K   Q   I   M   Q   820

AGG AAT GGT AAC AAC ATA TTG ACA AAC AGA ACA GGC CCC GTT AAC GCT CCG TAT ACG TTG CTG TTC CCA ACA AGT GAA GGT GGA  2602
 R   N   G   N   N   I   L   T   N   R   T   G   P   V   N   A   P   Y   T   L   L   F   P   T   S   E   G   G   848

CTT ACA GGC AAA GGA ATT CCA AAC AGT GTG TCA ATA TAG AAG AGT TTT GAG TAC ACA TGT AAA ATG TAA GAA AGC TGG AGT TTG  2686
 L   T   G   K   G   I   P   N   S   V   S   I  xxx                                                              861
                                             *

AAT GAA TCT TCA AAT AAA ATT GAT CAT TAC TGT ATG TTC ATT TCT CCT AAG TTT ACT GTA TTT TCT TTT CAA CCT TAC TTG TTA  2770

TGT AAT TCT CAG TAT GTT GTG AGA ATA ATA AAA CTA ATT CCA GCT GAA AAG TTT CAA TAT ATT TTG CAA TTA AAA AAA AAA AAA  2854

AAA AAA                                                                                                          2860
```

Figure 1a. (Legend on facing page.)

b

```
TT CTG TTT AAA TAG TTA ATC ATG TCT TTG GGT GGA ATT GTG GAT GCC ATC CTT GGA AAA GAT GAT AGG CCA AAA GTG AAA GGA  83
                            M   S   L   G   G   I   V   D   A   I   L   G   K   D   D   R   P   K   V   K   G   21

AGA GTG ATT TTG ATG AAA AAA AAT GTT CTA GAC TTC ATT AAT ATA GGT GCT TCA GTT GTT GAT GGC ATT TCT GAT TTA CTT GGC  167
 R   V   I   L   M   K   K   N   V   L   D   F   I   N   I   G   A   S   V   V   D   G   I   S   D   L   L   G   49

CAA AAA GTC TCT ATC CAA TTG ATA AGT GGT TCT GTT AAT TAT GAT GGT TTG GAA GGG AAA CTG AGC AAT CCA GCA TAC TTA GAG  251
 Q   K   V   S   I   Q   L   I   S   G   S   V   N   Y   D   G   L   E   G   K   L   S   N   P   A   Y   L   E   77

AGT TGG CTT ACA GAC ATC ACC CCA ATA ACA GCA GGG GAA TCA ACT TTT AGT GTT ACA TTT GAC TGG GAT CGT GAC GAG TTT GGA  335
 S   W   L   T   D   I   T   P   I   T   A   G   E   S   T   F   S   V   T   F   D   W   D   R   D   E   F   G   105

GTT CCA GGA GCA TTC ATC ATC AAG AAT CTT CAT CTT AAT GAG TTC TTT CTC AAG TCA CTC ACA CTC GAA GAT GTT CCT AAT TAT  419
 V   P   G   A   F   I   I   K   N   L   H   L   N   E   F   F   L   K   S   L   T   L   E   D   V   P   N   Y   133

GGA AAA ATC CAT TTT GTA TGC AAT TCT TGG GTT TAT CCT GCT TTT AGA TAC AAG TCT GAC CGC ATT TTC TTT GCC AAT CAG GCT  503
 G   K   I   H   F   V   C   N   S   W   V   Y   P   A   F   R   Y   K   S   D   R   I   F   F   A   N   Q   A   161

TAT CTC CCA AGT GAA ACA CCA CAA CCA TTG CGA AAA TAC AGA GAA AAT GAA CTG GTA GCT TTG CGA GGA GAT GGA ACT GGA AAG  587
 Y   L   P   S   E   T   P   Q   P   L   R   K   Y   R   E   N   E   L   V   A   L   R   G   D   G   T   G   K   189

CTT GAA GAA TGG GAC AGG GTT TAT GAT TAT GCT TGC TAC AAT GAC TTG GGT GAA CCA GAT AAG GGG GAA GAG TAT GCT AGG CCT  671
 L   E   E   W   D   R   V   Y   D   Y   A   C   Y   N   D   L   G   E   P   D   K   G   E   E   Y   A   R   P   217

ATC CTT GGA GGG TCC TCT GAG TAC CCG TAT CCT CGT AGA GGC AGG ACA GGC CGC GAA CCA ACC AAA GCA GAT CCT AAT TGC GAG  755
 I   L   G   G   S   S   E   Y   P   Y   P   R   R   G   R   T   G   R   E   P   T   K   A   D   P   N   C   E   245

AGC AGG AAC CCA TTG CCT ATG AGC TTA GAC ATA TAT GTC CCA AGG GAC GAG CGA TTT GGT CAT GTG AAG AAG TCA GAC TTT TTG  839
 S   R   N   P   L   P   M   S   L   D   I   Y   V   P   R   D   E   R   F   G   H   V   K   K   S   D   F   L   273

ACG TCG TCC TTA AAA TCC TCT TTG CAA ACG CTC CTC CCT GCG TTT AAG GCT TTG TGC GAT AAC ACG CCT AAT GAG TTC AAT AGC  923
 T   S   S   L   K   S   S   L   Q   T   L   L   P   A   F   K   A   L   C   D   N   T   P   N   E   F   N   S   301

TTT GCG GAT GTA CTT AAT CTC TAT GAA GGA GGA ATC AAG TTG CCT GAA GGC CCT TGG TTG AAA GCC ATT ACT GAT AAC ATT TCC  1007
 F   A   D   V   L   N   L   Y   E   G   G   I   K   L   P   E   G   P   W   L   K   A   I   T   D   N   I   S   329

TCA GAG ATA CTA AAA GAC ATC CTT CAA ACG GAT GGT CAA GGC CTA CTT AAG TAC CCA ACT CCT CAG GTT ATT CAA GGC GAT AAA  1091
 S   E   I   L   K   D   I   L   Q   T   D   G   Q   G   L   L   K   Y   P   T   P   Q   V   I   Q   G   D   K   357

ACT GCA TGG AGG ACG GAT GAA GAA TTT GGG AGA GAA ATG TTG GCA GGA TCC AAT CCT GTC TTA ATC AGT AGA CTC CAA GAA TTT  1175
 T   A   W   R   T   D   E   E   F   G   R   E   M   L   A   G   S   N   P   V   L   I   S   R   L   Q   E   F   385

CCT CCG AAG AGC AAG TTG GAT CCA ACC ATA TAT GGA AAC CAA AAC AGT ACA ATT ACC ACA GAA CAT GTA CAG GAT AAG TTG AAT  1259
 P   P   K   S   K   L   D   P   T   I   Y   G   N   Q   N   S   T   I   T   T   E   H   V   Q   D   K   L   N   413

GGA TTA ACA GTG AAT GAG GCA ATC AAG AGT AAC AGG TTA TTC ATA TTG AAC CAC CAT GAC ATC GTG ATG CCA CTA TTG AGG AAA  1343
 G   L   T   V   N   E   A   I   K   S   N   R   L   F   I   L   N   H   H   D   I   V   M   P   L   L   R   K   441

ATT AAC ATG TCA GCA AAC ACA AAA GCC TAT GCC TCA AGA ACT CTG CTC TTC CTA CAA GAT GAT AGA ACT TTG AAG CCA CTA GCA  1427
 I   N   M   S   A   N   T   K   A   Y   A   S   R   T   L   L   F   L   Q   D   D   R   T   L   K   P   L   A   469

ATT GAA CTA AGC TTG CCA CAT CCA GAC GGA GAT CAA TTT GGT ACT GTT AGT AAA GTA TAT ACA CCA GCT GAC CAA GGT GTT GAA  1511
 I   E   L   S   L   P   H   P   D   G   D   Q   F   G   T   V   S   K   V   Y   T   P   A   D   Q   G   V   E   497

GGT TCT ATC TGG CAG TTT GCC AAA GCC TAT GTA GCA GTG AAT GAC ATG GGC ATT CAT CAG CTT ATT AGC CAC TGG TTG AAT ACA  1595
 G   S   I   W   Q   F   A   K   A   Y   V   A   V   N   D   M   G   I   H   Q   L   I   S   H   W   L   N   T   525
                                                                                             ★
CAC GCG GTG ATC GAA CCA TTT GTG GTT GCA ACA AAT AGG CAT CTA AGT GTG CTT CAT CCC ATT CAT AAA CTT CTT CAT CCT CAT  1679
 H   A   V   I   E   P   F   V   V   A   T   N   R   H   L   S   V   L   H   P   I   H   K   L   L   H   P   H   553
 ★
TTC CGT AAC ACG ATG AAC ATA AAT GCT TTA GCA AGA GAG ACC TTG ACC TAT GAT GGT GGT TTT GAG ACG TCT CTT TTT CCT GCC  1763
 F   R   N   T   M   N   I   N   A   L   A   R   E   T   L   T   Y   D   G   G   F   E   T   S   L   F   P   A   581

AAA TAT TCC ATG GAA ATG TCA GCA GCA GCT TAC AAA GAT TGG GTT TTC CCT GAA CAA GCA CTT CCT GCT GAT CTC CTC AAA AGA  1847
 K   Y   S   M   E   M   S   A   A   A   Y   K   D   W   V   F   P   E   Q   A   L   P   A   D   L   L   K   R   609

GGA GTG GCT GTT GAG GAC TTG AGC TCC CCA CAT GGC ATT CGT TTA CTG ATT CTG GAC TAT CCA TAT GCT GTT GAT GGC TTG GAA  1931
 G   V   A   V   E   D   L   S   S   P   H   G   I   R   L   L   I   L   D   Y   P   Y   A   V   D   G   L   E   637

ATT TGG GCA GCA ATC AAA AGT TGG GTA ACA GAA TAT TGC AAG TTC TAT TAC AAA TCT GAC GAG ACA GTA GAG AAA GAC ACT GAA  2015
 I   W   A   A   I   K   S   W   V   T   E   Y   C   K   F   Y   Y   K   S   D   E   T   V   E   K   D   T   E   665

CTC CAA GCT TGG TGG AAG GAG CTC CGC GAA GAA GGA CAT GGC GAC AAG AAA GAT GAG GCT TGG TGG CCT AAA CTG CAA ACT CGA  2099
 L   Q   A   W   W   K   E   L   R   E   E   G   H   G   D   K   K   D   E   A   W   W   P   K   L   Q   T   R   693

CAA GAG CTC AGA GAT TGT TGC ACC ATC ATT ATA TGG ATA GCT TCA GCA CTT CAT GCA GCA CTC CAT TTT GGC TTA TAC TCT TAC  2183
 Q   E   L   R   D   C   C   T   I   I   I   W   I   A   S   A   L   H   A   A   L   H   F   G   L   Y   S   Y   721
                                                                     ★
GCT GGT TAT CTC CCT AAT CGC CCT ACT TTA AGC TGT AAT TTG ATG CCA GAG CCA GGA AGT GTT GAG TAT GAA GAG CTC AAG ACA  2267
 A   G   Y   L   P   N   R   P   T   L   S   C   N   L   M   P   E   P   G   S   V   E   Y   E   E   L   K   T   749

AAT CCA GAC AAG GTA TTC CTA AAA ACA TTT GTT CCT CAG TTG CAA TCA CTG CTT GAA ATT TCC ATC TTT GAG GTC TCG TCA AGG  2351
 N   P   D   K   V   F   L   K   T   F   V   P   Q   L   Q   S   L   L   E   I   S   I   F   E   V   S   S   R   777

CAT GCT TCA GAT GAG GTT TAC TTG GGA CAA AGG GAC TCA ATT GAA TGG ACA AAG GAT AAA GAA CCA CTT GTA GCT TTT GAG AGG  2435
 H   A   S   D   E   V   Y   L   G   Q   R   D   S   I   E   W   T   K   D   K   E   P   L   V   A   F   E   R   805

TTT GGA AAG ATG CTA AGT GAT ATC GAG AAT CGA ATT ATG ATA ATG AAT AGT CAT AAG AGT TGG AAG AAC AGG TCA GGG CCT GTT  2519
 F   G   K   M   L   S   D   I   E   N   R   I   M   I   M   N   S   H   K   S   W   K   N   R   S   G   P   V   833

AAC GTT CCA TAT ACG TTG CTC TTT CCC ACA AGT GAA GAG GGA CTC ACA GGC AAA GGA ATT CCC AAC AGT GTG TCT ATA TAG AAC  2603
 N   V   P   Y   T   L   L   F   P   T   S   E   E   G   L   T   G   K   G   I   P   N   S   V   S   I  xxx      860
                                                                                                     ★
TTA TTA TTC AAT CAG TTT GTT GTG CTT GTG TTA CTT GTT ATT CCC AAC CAA ATA AAC TCT TTG TTC CAA ATA AAG AGT ATT GTC  2687

TTG TAT TGT CTT GTG TGT TGT GTT GTA TTG TAT TAT ATT GTA TAG TAT TAT TGA TTT AAA AAA AAA AAA AAA  2759
```

Figure 1. Nucleotide sequence and deduced amino acid sequence of *tomloxA* (a, facing page) and *tomloxB* (b) genes. The four amino acid sequences determined by peptide sequencing of isolated lipoxygenase are underlined. The two regions chosen for degenerate oligonucleotide PCR primers are shaded. (★) represents the four iron ligands (Boyington et al., 1993a, 1993b; Minor et al., 1993).

SDS-PAGE of the purified membrane-associated protein indicated an apparent molecular mass of 97 kD (Bowsher et al., 1992). Together with preliminary RNA blot analysis, this suggested that the lipoxygenase transcripts are around 3 kb. The library was rescreened with a 5′ fragment of the *tomloxA* clone, but a larger cDNA was not detected.

To obtain the 5′ ends of *tomloxA* and *tomloxB*, genomic clones (see below) were used. The genomic clone corresponding to *tomloxB* contained the 5′ end of the gene, whereas the genomic clone of *tomloxA* was found to be missing approximately 250 bp of coding region. Because the RACE procedure (Froham, 1990) was unsuccessful using primers specific to the cDNA clones, antisense primers closer to the 5′ end of the mRNA were designed based on exon sequence within the *tomloxA* genomic clone. First-strand fruit cDNA was polyadenylated and amplified with these primers and the RACE adapter and poly(T)-containing primers to produce a 330-bp PCR fragment. A sense primer specific to the 5′ end of this fragment was then used with a *tomloxA* antisense primer to amplify from the fruit cDNA a fragment that overlapped with *tomloxA*. These fragments were cloned together to produce a full-length *tomloxA* cDNA (Fig. 1a).

The start codon for *tomloxB* was deduced from the corresponding genomic clone. There is an in-frame stop codon upstream of this ATG, and all other prospective ATG codons are followed by in-frame stop codons. Also, the deduced amino acid sequence within this region is similar to that found in other plant lipoxygenase N termini (Fig. 2a). A sense primer that included the putative ATG was designed and used with a *tomloxB* antisense primer to amplify a 1.3-kb fragment from fruit cDNA. These overlapping fragments were cloned to produce the full-length *tomloxB* (Fig. 1b).

Analysis of Genomic Clones

A λ FixII tomato genomic library was screened with fragments of the two cDNAs. Six positive clones were isolated and restriction fragments were identified in the genomic clones that corresponded to those identified in the genomic blots. One of these clones was partially sequenced and found to contain the complete sequence of the gene corresponding to *tomloxB* as well as the 3′ end of a closely related gene. Preliminary results from the analysis of a second genomic clone indicated the presence of portions of these same two genes. The inserts of three other genomic clones were similarly analyzed to obtain the complete sequence of the gene corresponding to *tomloxA*.

Sequence Analysis of Lipoxygenase cDNAs

The nucleotide and derived amino acid sequences of the two tomato lipoxygenase cDNAs are presented in Figure 1. The 2860-nucleotide sequence of *tomloxA* (Fig. 1a) consists of a 58-bp 5′ untranslated region, a 2580-bp open reading frame, a stop codon, and a 219-bp 3′ untranslated region including an 18-bp poly(A) tail. The derived amino acid sequence of 860 amino acids has a predicted molecular mass of 96.8 kD. The amino acid sequences of the four purified peptides were found within this sequence (underlined in Fig. 1a). In Figure 1b, the 2759-nucleotide sequence of *tomloxB* is shown. The open reading frame of 2577 bp predicts an 859-amino acid protein with a molecular mass of 97.1 kD. The 3′ untranslated region is 162 bp including a 15-bp poly(A) tail. The amino acid sequence of *tomloxB* was found to differ at several residues from the purified peptides. Although the purified lipoxygenase was associated with thylakoid membranes, there was no evidence of transit peptide sequence in either cDNA.

At the DNA level the two tomato lipoxygenase genes show 74% homology, and at the amino acid level they show 72% identity. The sequences were compared to nine plant lipoxygenase sequences available from GenBank (refs. in Fig. 2 legend) and a partial tobacco amino acid sequence (Bookjans et al., 1988). Nucleic acid sequence homology values ranged from 54 to 65%. At the amino acid level, identity values for *tomloxA* ranged from 56% with rice to 81% with tobacco (values on right in Fig. 2b). Values were similar but slightly lower (by 1–3%) for *tomloxB* and the other plant sequences except for the tobacco sequence, which shared 72% identity. There was little difference with either tomato sequence compared to the various lipoxygenase forms of soybean or pea; therefore, a representative sequence was used for each in Figure 2b.

The sequences of N termini of lipoxygenases differ markedly (Siedow, 1991). Soybean lipoxygenase-1 has about 30 fewer residues than other plant lipoxygenases in this region. The comparison of the first 100 residues in Figure 2b demonstrates this dissimilarity in contrast to the remaining regions. However, following the N-terminal 7 to 20 residues, there is a 17-amino acid region within the N termini that is highly conserved, with most substitutions being conservative (Fig. 2a).

There are three important regions of sequence similarity in lipoxygenases. The C-terminal 8 amino acids are highly conserved (GIPNSISI for all the legumes and rice and GIPNSVSI for *Arabidopsis* and both tomato lipoxygenases). Tobacco is an exception to this (Bookjans et al., 1988). All of the plant and mammalian lipoxygenase sequences share a highly conserved region of 38 amino acids (region III in Fig. 2b). This region spans amino acids 516 to 553 in *tomloxA* and *tomloxB*. There are 13 completely conserved residues in this region (Steczko et al., 1992) that are also found in both tomato sequences. The five conserved His's were suggested as possible iron ligands. There is a sixth conserved His about 160 residues downstream in all sequences (712 in *tomloxA*, 711 in *tomloxB*). This His is situated in a region of about 13 residues that are highly conserved in plant lipoxygenases and mammalian 5-lipoxygenases (Siedow, 1991), and it has also been suggested as a putative iron binding site. Site-specific mutations of the six His's in soybean lipoxygenase-1 showed that H499, H504, and H690 (H521, H526, H712 in *tomloxA*) were necessary for enzyme activity and iron binding (Steczko et al., 1992; Steczko and Axelrod, 1992). Recent crystallographic determinations have revealed the three-dimensional structure of lipoxygenase (soybean lipoxygenase-1) and suggest the importance of many of the conserved residues in iron binding and enzyme activity (Boyington et al., 1993a, 1993b; Minor et al., 1993). Minor et al. (1993) identified five ligands: H499, H504, H690, the C-terminal carboxylate group of I839, and N694. The latter residue is conserved in all of

a

LOX1	7	K I K **G** T V V L **M** P **K** **N** E L E V N
LOX2	18	K I K **G** T V V L **M** R **K** **N** V L D F N
LOX3	11	K I K **G** T V V L **M** R **K** **N** V L D V N
SC5	10	K I K **G** T V V L **M** P **K** **N** V L D F N
PEA2	14	K I R **G** T V V L **M** R **K** **N** V L D F N
PEA3	14	K I K **G** T V V L **M** R **K** **N** V L D I N
BEAN	10	K I K **G** T V V L **M** T **K** **N** V F D F N
RICE	16	R L K **G** S L V L **M** R **K** **N** A L D I N
ARAB	20	K V K **G** T V V L **M** K **K** **N** V L D F N
TOMA	18	K V K **G** T V V M **M** K **K** **N** A L D F T
TOMB	18	K V K **G** R V I L **M** K **K** **N** V L D F I

b

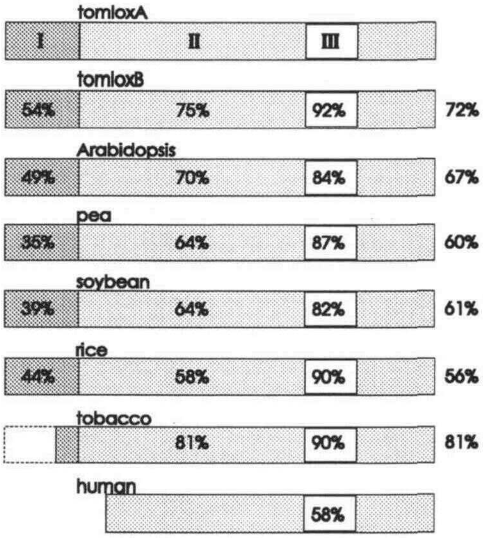

Figure 2. Lipoxygenase amino acid sequence comparisons. a, Comparison of sequences of a 17-amino acid region from N termini of plant lipoxygenases. LOX1, Soybean lipoxygenase-1 (Shibata et al., 1987); LOX2, soybean lipoxygenase-2 (Shibata et al., 1988); LOX3, soybean lipoxygenase-3 (Yenofsky et al., 1988); SC5, soybean cotyledon lipoxygenase (Shibata et al., 1991); PEA2, pea seed lipoxygenase-2 (Ealing and Casey, 1989); PEA3, pea seed lipoxygenase-3 (Ealing and Casey, 1988); BEAN, French bean lipoxygenase (A.J. Slusarenko, unpublished data); RICE, rice seed lipoxygenase L-2 (Ohta et al., 1992); ARAB, *Arabidopsis* lipoxygenase (Melan et al., 1993); TOMA, TOMB, this paper. Boldface letters refer to invariant amino acids; numbers indicate distance from N-terminal M. b, Comparison of deduced amino acid sequence identity of lipoxygenase genes with *tomloxA*. Region I, first 100 residues; region II, from amino acid 100 to C terminal; region III, the 38-residue conserved region (Steczko et al., 1992)—amino acids 516–553 in *tomloxA*. Numbers on the right represent overall identity.

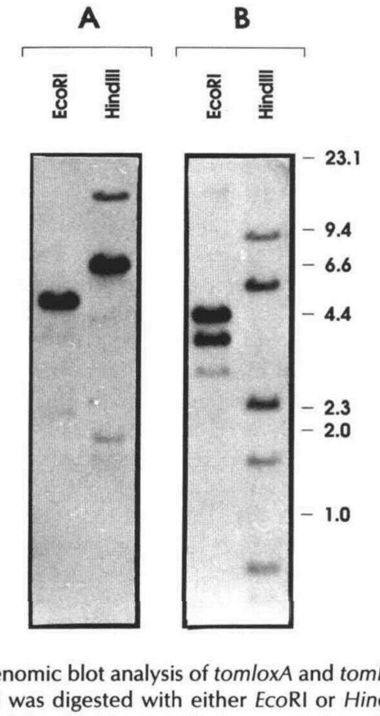

Figure 3. Genomic blot analysis of *tomloxA* and *tomloxB*. Genomic DNA (10 μg) was digested with either *Eco*RI or *Hin*dIII. The same blot was hybridized with a 1.4-kb fragment of *tomloxA* cDNA (A) or a 1.5-kb fragment of *tomloxB* cDNA (B). Washes were in 0.1× SSC at 60°C. Size markers (kb) are indicated on the right.

the plant sequences including *tomloxA* (N716) but is substituted for by His in *tomloxB* (H715) and some of the mammalian lipoxygenases. This is assumed to be a functional substitute (Minor et al., 1993). These residues have been marked in Figure 1. Boyington et al. (1993a, 1993b) determined that the iron was coordinated to four ligands (the three His's and the C-terminal Ile) and that there were two adjacent, unoccupied positions, with N694 being very close to an unoccupied coordination position.

Genomic Blot Analysis

To evaluate the number of lipoxygenase genes in the tomato genome, Southern hybridization of tomato genomic DNA was performed with the 3′ ends of *tomloxA* and *tomloxB* cDNAs. At low stringency (2× SSC, 60°C), both probes hybridized with various intensities to a common set of 8 and 13 fragments for the *Eco*RI and *Hin*dIII digests, respectively, with sizes ranging from 0.8 to 12 kb (data not shown). At higher stringencies (0.1× SSC, 60 and 65°C), the probe from *tomloxA* hybridized most strongly to single *Eco*RI and *Hin*dIII fragments and hybridized moderately to two other *Hin*dIII fragments (Fig. 3). A different set of bands was observed with the probe from *tomloxB*. When washed in 0.1× SSC at 60°C, it hybridized most strongly to two *Eco*RI fragments and moderately to a third fragment. It also hybridized strongly to two *Hin*dIII fragments and moderately to three other fragments (Fig. 3). When the stringency was increased further (0.1× SSC, 65°C), *tomloxB* hybridized most strongly to a single band and moderately to a second band for the *Eco*RI digest and hybridized moderately to two bands for the *Hin*dIII digest (data not shown).

These results suggest that tomato lipoxygenase is encoded by a family of at least three genes. Two of these genes can be associated with the *tomloxA* and *tomloxB* cDNAs characterized in this paper. In addition, a putative third gene highly related to the *tomloxB* cDNA was detected when washing was at a moderate stringency (Fig. 3). Extra bands observed at lower stringencies also suggest the presence of similar sequences that could putatively encode a fourth, less closely related gene. The genomic clones confirm the presence of at least three genes in the genome.

Expression of Lipoxygenase Genes

To determine the expression patterns of the two tomato lipoxygenase genes during fruit ripening, total RNA was isolated from pericarp at four ripening stages (immature green, mature green, breaker, and ripe) and subjected to RNA blot analysis. This was repeated for other tissue (seed, germinated seed, leaf, and root) to determine whether the genes were fruit specific. As seen in Figure 4a, during fruit ripening *tomloxA* mRNA first appeared in mature green fruit, increased to a maximum at breaker stage, and was not evident in ripe fruit. The pattern was different for the *tomloxB* message, which was first seen in breaker fruit but reached its highest level in ripe fruit. In the course of several experiments the patterns of expression did not vary. However, considering differences in probe-specific activities and exposure times, the levels of *tomloxA* and *tomloxB* are probably similar at their respective peaks. In other tissues transcript for *tomloxB* was undetectable (Fig. 4b). *tomloxA* message was faintly expressed in seeds that had imbibed, it was more strongly expressed in germinating seeds, and it was undetectable in leaf or root. Neither transcript was seen in pistils, anthers, or senescing leaves (data not shown). In each case the transcript observed was approximately 3 kb.

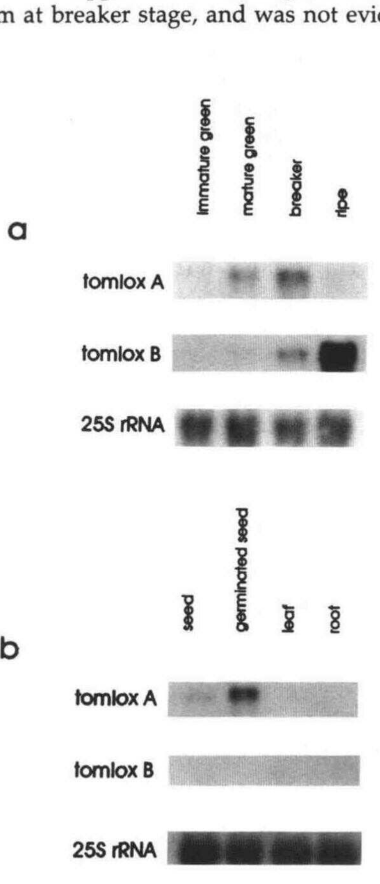

Figure 4. Differential expression of *tomloxA* and *tomloxB*. Total RNA (20 μg) from pericarp at four stages of ripening (a) and from four other sources of tissue (b). The blots were hybridized with each of the full-length cDNAs and a wheat ribosomal RNA probe. Washes were in 0.1× SSC at 65°C for the lipoxygenase clones and 0.2× SSC at 55°C for the ribosomal clone.

DISCUSSION

We have isolated and sequenced two full-length lipoxygenase cDNA clones from tomato. As evidenced by amino acid sequence data, one of these (*tomloxA*) encodes a lipoxygenase that was purified from breaker-stage fruit. This enzyme had been found through several lines of evidence to be associated with thylakoid membranes (Bowsher et al., 1992). The deduced amino acid sequences for the two genes are very similar (72% identity) to each other and to other plant lipoxygenases. Although the lipoxygenases are most divergent at the N termini, *tomloxA* displays enough similarity to the others to indicate the lack of a chloroplast transit peptide. Analysis of the genomic clones confirmed the translation start codon for *tomloxA*, thus excluding the possibility of peptide sequence beginning farther upstream.

Nuclear-encoded chloroplast proteins are thought to require a cleavable, N-terminal transit peptide for transport into the chloroplast (Berry-Lowe and Schmidt, 1991; De Boer and Weisbeek, 1991). At least three exceptions to this have been published. A heat-shock protein from *Chlamydomonas* (Grimm et al., 1989), a spinach chloroplast membrane protein (Salomon et al., 1990), and most recently, a stromal betaine aldehyde dehydrogenase from spinach and sugar beet (Rathinasabapathi et al., 1994) all lack typical transit peptides. There is a possibility that the tomloxA protein is targeted to the choloroplast using information in the mature protein.

During ripening of tomato fruit, chloroplasts develop into chromoplasts and at an early stage in this process a massive reordering of plastid structure occurs. This is characterized by the disappearance of Chl pigments, the disintegration of the intricate thylakoid membrane system, and the accumulation of the carotenoid lycopene in less elaborate membranous structures and within the chromoplast envelope (Cheung et al., 1993; Lawrence et al., 1993). Thylakoid membranes contain a high amount of polyunsaturated fatty acids. In some species up to 95% of the total fatty acids in these membranes is linolenic acid (Douce and Joyard, 1991). Although the cues for the chloroplast-chromoplast transition are unknown, the nuclear genome is known to be involved and there is recent evidence that there is synthesis and import of new proteins during the transition (Lawrence et al., 1993). Perhaps lipoxygenase is involved in this transition.

There also exists the possibility that *tomloxA* lipoxygenase is not associated with thylakoid membranes in vivo, given the uncertainties of cell fractionation experiments, although thylakoids isolated from intact chloroplasts were used in the immunological identification of the enzyme (Bowsher et al., 1992). Lipoxygenases could be associated with membranes (other than thylakoids) in other ways, including through the action of another protein, as was outlined for the mammalian 5-lipoxygenase and FLAP (Mancini et al., 1993). The recent crystallographic studies have shown the structure of soybean lipoxygenase-1 to contain two domains (Boyington et al., 1993). The first domain comprises the 146 N-terminal residues, which form a barrel that has an interior of densely packed hydrophobic side chains. It is interesting to note that this domain is identical in connectivity to a similar barrel found in human pancreatic lipase, which is involved in colipase binding. Domain I is separate from the rest of the molecule, making only loose contact, and is not present in mammalian lipoxygenases. This region could possibly be involved in lipoxygenase binding to a membrane or to a membrane protein.

The two tomato lipoxygenase genes display different expression patterns during development and fruit ripening. *tomloxA* is expressed in germinating seeds (found to be a site of high lipoxygenase activity in other studies [Siedow, 1991]). In fruit, *tomloxA* is expressed most strongly in the breaker stage, whereas *tomloxB* mRNA is at its highest level in ripe fruit. *tomloxB* appears to be fruit specific, whereas *tomloxA* may have different functions in different tissues. The significance of these expression patterns is unknown. As mentioned, ripening and senescence involve the loss of membrane integrity, and it has been proposed that lipoxygenase is involved in this process. Perhaps the product of *tomloxA* contributes to changes in membrane structure that allow changes in metabolite distribution during ripening (and germination). The lipoxygenase isoform of *tomloxB* may be more specific to the degradative process of fruit senescence. Another possible mechanism for the involvement of lipoxygenase in tomato ripening is its role in the biosynthesis of jasmonic acid. This growth regulator has been shown to play a role in senescence with exogenous applications.

Lipoxygenase is also thought to be important in the formation of flavors and odors (Mack et al., 1987; Hildebrand, 1989). The fatty acid hydroperoxides that are the primary products of the lipoxygenase reaction and that can be damaging to membranes can be converted to less damaging products. The characteristic odor of tomato is in part due to the volatile aldehyde hexenal, which results from the cleavage of the 13-linoleic acid hydroperoxide (Mack et al., 1987). *tomloxB* may be more specifically involved in this reaction.

Our findings further implicate lipoxygenase in the process of ripening/senescence. Further study is necessary to determine the nature of the membrane association of the lipoxygenase encoded by *tomloxA* and to definitively demonstrate its subcellular localization. This information would help elucidate the specific role of lipoxygenase in ripening and in various other physiological functions in which it has been implicated.

ACKNOWLEDGMENT

We are grateful to Rajeev Rastogi for providing fruit RNA samples.

Received January 18, 1994; accepted May 19, 1994.
Copyright Clearance Center: 0032-0889/94/106/0109/10.
The GenBank accession numbers for the sequences reported in this article are U09026 (*tomloxA*) and U09025 (*tomloxB*).

LITERATURE CITED

Bell E, Mullet JE (1991) Lipoxygenase gene expression is modulated in plants by water deficit, wounding, and methyl jasmonate. Mol Gen Genet **230:** 456–462

Berry-Lowe SL, Schmidt GW (1991) Chloroplast protein transport. *In* L Bogorad, IK Vasil, eds, The Molecular Biology of Plastids: Cell Culture and Somatic Cell Genetics of Plants, Vol 7A. Academic Press, San Diego, CA, pp 257–302

Bookjans G, Altschuler M, Brockman J, Yenofsky R, Polacco J, Dickson R, Collins G, Hildebrand D (1988) Molecular biological studies of plant lipoxygenases. *In* TH Applewhite, ed, Proceedings of the World Conference on Biotechnology for the Fat and Oil Industry. American Oil Chemical Society Press, Urbana, IL, pp 301–304

Bowsher CG, Ferrie BJM, Ghosh S, Todd J, Thompson JE, Rothstein SJ (1992) Purification and partial characterization of a membrane-associated lipoxygenase in tomato fruit. Plant Physiol **100:** 1802–1807

Boyington JC, Gaffney BJ, Amzel LM (1993a) Structure of soybean lipoxygenase-1. Biochem Soc Trans **21:** 744–748

Boyington JC, Gaffney BJ, Amzel LM (1993b) The three-dimensional structure of an arachidonic acid 15-lipoxygenase. Science **260:** 1482–1486

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep **11:** 113–116

Cheung AY, McNellis T, Piekos B (1993) Maintenance of chloroplast components during chromoplast differentiation in the tomato mutant green flesh. Plant Physiol **101:** 1223–1229

Croft KPC, Jüttner F, Slusarenko AJ (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol **101:** 13–24

De Boer AD, Weisbeek PJ (1991) Chloroplast protein topogenesis: import, sorting and assembly. Biochim Biophys Acta **1071:** 221–253

Douce R, Joyard J (1991) Structure, organization, and properties of plastid envelope membranes. *In* L Bogorad, IK Vasil, eds, The Molecular Biology of Plastids: Cell Culture and Somatic Cell Genetics of Plants, Vol 7A. Academic Press, San Diego, CA, pp 217–256

Ealing PM, Casey R (1988) The complete amino acid sequence of a pea (*Pisum sativum*) seed lipoxygenase predicted from a near full-length cDNA. Biochem J **253:** 915–918

Ealing PM, Casey R (1989) The cDNA cloning of a pea (*Pisum sativum*) seed lipoxygenase: sequence comparisons of the two major pea seed lipoxygenase isoforms. Biochem J **264:** 929–932

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Feußner I, Kindl H (1992) A lipoxygenase is the main lipid body protein in cucumber and soybean cotyledons during the stage of triglyceride mobilization. FEBS Lett **298:** 223–225

Fournier J, Pouénat M, Rickauer M, Rabinovitch-Chable H, Rigaud M, Esquerré-Tugayé M (1993) Purification and characterization of elicitor-induced lipoxygenase in tobacco cells. Plant J **3:** 63–70

Frohman MA (1990) RACE: rapid amplification of cDNA ends. *In* MA Innis, DH Gelfand, JJ Sninsky, TJ White, eds, PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, pp 28–38

Goring DR, Banks P, Beversdorf WD, Rothstein SJ (1992) Use of the polymerase chain reaction to isolate an S-locus glycoprotein cDNA introgressed from *Brassica campestris* into *B. napus* ssp. *oleifera*. Mol Gen Genet **234:** 185–192

Grimm B, Ish-Shalom D, Even D, Glaczinski H, Ottersbach P, Ohad I, Kloppstech K (1989) The nuclear-coded chloroplast 22-kDa heat-shock protein of *Chlamydomonas*: evidence for translocation into the organelle without a processing step. Eur J Biochem **182:** 539–546

Harvey RJ, Darlison MG (1991) Random-primed cDNA synthesis facilitates the isolation of multiple 5′ cDNA ends by RACE. Nucleic Acids Res **19:** 4002

Hildebrand DF (1989) Lipoxygenases. Physiol Plant **76:** 249–253

Kato T, Ohta H, Tanaka K, Shibata D (1992) Appearance of new lipoxygenases in soybean cotyledons after germination and evidence for expression of a major new lipoxygenase gene. Plant Physiol **98:** 324–330

Lawrence SD, Cline K, Moore GA (1993) Chromoplast-targeted proteins in tomato (*Lycopersicon esculentum* Mill.) fruit. Plant Physiol **102:** 789–794

Lee CC, Caskey CT (1990) cDNA cloning using degenerate primers. *In* MA Innis, DH Gelfand, JJ Sninsky, TJ White, eds, PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, pp 46–53

Lynch DV, Thompson JE (1984) Lipoxygenase-mediated production of superoxide anion in senescing plant tissue. FEBS Lett **173:** 251–254

Mack AJ, Peterman TK, Siedow JN (1987) Lipoxygenase isozymes in higher plants: biochemical properties and physiological role. Isozymes Curr Top Biol Med Res **13:** 127–154

Mancini JA, Abramovitz M, Cox ME, Wong E, Charleson S, Perrier H, Wang Z, Prasit P, Vickers PJ (1993) 5-Lipoxygenase-activating protein is an arachidonate binding protein. FEBS Lett **318:** 277–281

Matsui K, Irie M, Kajiwara T, Hatanaka A (1992) Developmental changes in lipoxygenase activity in cotyledons of cucumber seedlings. Plant Sci **85:** 23–32

Melan MA, Dong X, Endara ME, Davis KR, Ausubel FM, Peterman TK (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol **101:** 441–450

Minor W, Steczko J, Bolin JT, Otwinowski Z, Axelrod B (1993) Crystallographic determination of the active site iron and its ligands in soybean lipoxygenase L-1. Biochemistry **32:** 6320–6323

Ohta H, Shirano Y, Tanaka K, Morita Y, Shibata D (1992) cDNA cloning of rice lipoxygenase L-2 and characterization using an active enzyme expressed from the cDNA in *Escherichia coli.* Eur J Biochem **206:** 331–336

Paliyath G, Droillard MJ (1992) The mechanisms of membrane deterioration and disassembly during senescence. Plant Physiol Biochem **30:** 789–812

Rastogi R, Dulson J, Rothstein SJ (1993) Cloning of tomato (*Lycopersicon esculentum* Mill.) arginine decarboxylase gene and its expression during fruit ripening. Plant Physiol **103:** 829–834

Rathinasabapathi B, McCue KF, Gage DA, Hanson AD (1994) Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. Planta **193:** 155–162

Rouet-Mayer M, Bureau J, Laurière C (1992) Identification and characterization of lipoxygenase isoforms in senescing carnation petals. Plant Physiol **98:** 971–978

Salomon M, Fischer K, Flügge U, Soll J (1990) Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. Proc Natl Acad Sci USA **87:** 5778–5782

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schewe T, Kühn H (1991) Do 15-lipoxygenases have a common biological role? Trends Biochem Sci **16:** 369–373

Shibata D, Kato T, Tanaka K (1991) Nucleotide sequence of a soybean lipoxygenase gene and the short intergenic region between an upstream lipoxygenase gene. Plant Mol Biol **16:** 353–359

Shibata D, Steczko J, Dixon JE, Andrews PC, Hermodson M, Axelrod B (1988) Primary structure of soybean lipoxygenase-2. J Biol Chem **263:** 6816–6821

Shibata D, Steczko J, Dixon JE, Hermodson M, Yazdanparast R, Axelrod B (1987) Primary structure of soybean lipoxygenase-1. J Biol Chem **262:** 10080–10085

Siedow JN (1991) Plant lipoxygenase: structure and function. Annu Rev Plant Physiol Plant Mol Biol **42:** 145–188

Steczko J, Axelrod B (1992) Identification of the iron-binding histidine residues in soybean lipoxygenase L-1. Biochem Biophys Res Commun **186:** 686–689

Steczko J, Donoho GP, Clemens JC, Dixon JE, Axelrod B (1992) Conserved histidine residues in soybean lipoxygenase: functional consequences of their replacement. Biochemistry **31:** 4053–4057

Thompson JE (1988) The molecular basis for membrane deterioration during senescence. *In* LD Noodén, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, San Diego, CA, pp 51–83

Todd JF, Paliyath G, Thompson JE (1990) Characteristics of a membrane-associated lipoxygenase in tomato fruit. Plant Physiol **94:** 1225–1232

Vick BA, Zimmerman DC (1987) Oxidative systems for modification of fatty acids: the lipoxygenase pathway. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9. Lipids: Structure and Function. Academic Press, Orlando, FL, pp 53–90

Yamamoto S (1992) Mammalian lipoxygenases: molecular structures and functions. Biochim Biophys Acta **1128:** 117–131

Yenofsky RL, Fine M, Liu C (1988) Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3 gene. Mol Gen Genet **211:** 215–222

Zintz CB, Beebe DC (1991) Rapid re-amplification of PCR products purified in low melting point agarose gels. BioTechniques **11:** 158–162